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(54) Title: SCREENING FOR PHOSPHATIDYLINOSITOL RELATED-KINASE INHIBITORS

(57) Abstract

A method for identifying a compound inhibiting the phosphorylation activity of a phosphoinositide 3–kinase related kinase polypeptide is described. Antibodies having specific binding affinity for a conjugate including wortmannin are also described.

SCREENING FOR PHOSPHATIDYLINOSITOL
RELATED-KINASE INHIBITORS

5   Statement as to Federally Sponsored Research
        Funding for the work described herein was provided
by the federal government, which has certain rights in
the invention.

                Background of the Invention
10   The four mammalian members of the
phosphatidylinositol kinase-related kinase (PIKK) family
function in the regulation of eukaryotic cell-cycle
progression and cell-cycle checkpoint functions. Cell-
cycle checkpoints ensure that critical events such as DNA
15 replication and chromosome segregation are completed in a
timely and accurate fashion during each eukaryotic cell
cycle. In addition, certain checkpoints are activated by
environmental insults that result in DNA damage, such as
ionizing or ultraviolet radiation. Checkpoint activation
20 triggers signal transduction cascades that arrest the
progression through the cell cycle to allow repair of the
damaged DNA or initiation of programmed cell death. The
importance of cell cycle checkpoints in the maintenance
of genomic stability is highlighted by the clinical
25 syndrome ataxia-telangiectasia (AT), which results from
homozygous loss of function mutations in the ataxia-
telangiectasia mutated (ATM) gene. The gene product of
the ATM gene, ATM protein, is a member of the PIKK
family. AT patients are hyper-sensitive to ionizing
30 radiation and suffer from progressive cerebellar
degeneration, immunodeficiency, and a dramatically
increased incidence of various cancers, particularly
lymphomas. Cells derived from AT patients exhibit
defects in G1, S and G2 checkpoints following exposure to

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ionizing radiation. These cell cycle checkpoint defects
are thought to be causally related to the radiation
hypersensitivity and the high mutation rates displayed by
AT cells.

5   The ATaxia and Rad3 related protein (ATR), another
member of the PIKK family, shares some checkpoint control
functions with ATM, since overexpression of ATR corrects
the defective S-phase checkpoint in AT fibroblasts.
Cells deficient in ATR kinase activity are also more
10 sensitive to ionizing radiation and exhibit defects in
the G2 checkpoint similar to AT cells. Likewise, cells
deficient in the catalytic subunit of DNA-dependent
protein kinase (DNA-PK$_{cs}$), another member of the PIKK
family, are also hypersensitive to ionizing radiation and
15 exhibit a prolonged G2 arrest following irradiation.
DNA-PK$_{cs}$ forms a heterotrimer with Ku70 and Ku80 which is
critical for DNA double strand break repair in cells
exposed to ionizing radiation and in normal hematopoietic
cells undergoing V(D)J gene rearrangements. Unlike the
20 other members of the mammalian PIKK family, rapamycin
target protein or mammalian target of rapamycin (mTOR)
appears to participate in mitogenic signal transduction.
Mammalian TOR protein is also named FRAP or RAFT1.
Brown, E.J. et al., Nature 369:756 (1994); Subatini, M.
25 et al., Cell, 78:35 (1994).

                    Summary of the Invention
          The invention is based on the ability of
phosphoinositide 3-kinase related kinase (PIKK)
polypeptides to phosphorylate PHAS-1 protein. Assays for
30 identifying agents that inhibit the phosphorylation
activity of PIKK polypeptides are described. Such
inhibitors have therapeutic applications in
transplantation, cancer, and other proliferative
disorders.

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The invention relates to a method for identifying
a compound inhibiting the phosphorylation activity of a
PIKK polypeptide. The method includes incubating
isolated PIKK polypeptide and a substrate of the PIKK
5   polypeptide with the compound to determine if
phosphorylation of the substrate is inhibited. The PIKK
polypeptide can be, for example, mTOR, ataxia-
telangiectasia mutated protein or Ataxia and Rad3 related
protein. PHAS-I protein is a particularly useful
10 substrate of the PIKK polypeptides. As used herein, a
"compound" refers to a biological macromolecule such as
an oligonucleotide or a peptide, a chemical compound, a
mixture of chemical compounds, or an extract isolated
from bacterial, plant, fungal or animal matter. In
15 particular embodiments, suitable compounds induce
radioresistant DNA synthesis in irradiated cells
containing the compound.

The invention also features an antibody or
fragment thereof having specific binding affinity for a
20 conjugate including wortmannin or an analog thereof and a
polypeptide. The antibody can be polyclonal or
monoclonal. The polypeptide can be, for example, mTOR,
DNA-PK, ataxia-telangiectasia mutated protein or Ataxia
and Rad3 related protein.

25   The invention also relates to a method for
identifying a compound that induces radioresistant DNA
synthesis within cells. The method includes irradiating
the cells, wherein the cells include an effective amount
of the compound, and measuring radioresistant DNA
30 synthesis of the cell. The presence or absence of
radioresistant DNA synthesis is correlated with activity
of the compound.

Unless otherwise defined, all technical and
scientific terms used herein have the same meaning as
35 commonly understood by one of ordinary skill in the art

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to which this invention belongs. Although methods and
materials similar or equivalent to those described herein
can be used to practice the invention, suitable methods
and materials are described below. All publications,
5 patent applications, patents, and other references
mentioned herein are incorporated by reference in their
entirety. In case of conflict, the present
specification, including definitions, will control. In
addition, the materials, methods, and examples are
10 illustrative only and not intended to be limiting. Other
features and advantages of the invention will be apparent
from the following detailed description, and from the
claims.

Brief Description of the Drawings

15   Figure 1 depicts the eIF-4E binding activity of
phosphorylated PHAS-I. Radioactivity bound to PHAS-I in
each sample lane was quantitated and normalized to the
nonphosphorylated control (lane 1). The concentration of
wortmannin used in the pretreatment is given in μM.

20   Figure 2 depicts the phosphorylation of PHAS-1 by
immunoprecipitated ATM. Figure 2A depicts ^{32}P
incorporation into PHAS-I substrate from A549 lung
adenocarcinoma cells and GM 02052 fibroblasts null for
ATM expression. Results shown are the mean of two
25 experiments (error bars = s.d.). Figure 2B depicts
kinase activity of immunoprecipitated ATM and DNA-PK
washed in either kinase buffer (black bar) or high-salt
buffer (white bar) toward PHAS-I. Activity was
normalized to the kinase buffer washed sample. The mean
30 relative activity from two experiments is shown (error
bars = s.d.).

       Figures 3A-3C depict the covalent modification and
inhibition of ATM, DNA-PK and ATR, respectively, by

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wortmannin. Kinase activity was normalized to 1.0 for 0
nM wortmannin treatment. The mean relative activity from
two experiments for each kinase is plotted (error bars =
s.d.).

5   Figures 4A-4C depict the inhibition of covalent
modification of ATM, DNA-PK and ATR, respectively, by
wortmannin in intact cells. Kinase activity was
normalized to 1.0 for 0 μM wortmannin treatment. The
mean relative activity from two experiments for each
10 kinase is plotted. (error bars = s.d.).

Figure 5 depicts wortmannin-induced
radiosensitization of A549 cells as measured by a
clonogenic assay. Log-phase cells were exposed to graded
doses of radiation and then incubated with DMSO($\bullet$), 2
15 μM($\blacktriangledown$), 10 μM ($\triangledown$) or 20 μM ($\blacklozenge$) wortmannin for 14 days
prior to fixation and staining. Surviving fraction was
calculated relative to the appropriate drug treated,
unirradiated control. Results plotted are the mean of
four experiments (error bars = SEM).

20   Figure 6 depicts the effect of wortmannin on
radiation-induced G_2-phase delay in A549 cells
synchronized in S-phase by treatment with aphidicolin.
Histograms of red fluorescence intensity (DNA content)
from 20,000 ungated events are shown from a
25 representative experiment. The numbers in each panel
indicate the percentage of G_2-M cells in a test
population.

Figure 7 depicts the induction of radioresistant
DNA synthesis by wortmannin. DMSO ($\bullet$) or 3 μM ($\blacktriangledown$), 30 μM
30 ($\triangledown$) or 100 μM ($\blacklozenge$) wortmannin were added to log-phase A549
cells immediately prior to exposure to various doses of
irradiation. ^3H incorporation was normalized for each
treatment to the appropriate drug treated, unirradiated
control. Results plotted are the mean of three
35 experiments (error bars = SEM).

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Detailed Description

Dysfunction of certain PIKKs, including ATM, DNA-PK, and possibly ATR, leads to defects in DNA damage repair and hypersensitivity to ionizing radiation. As such, PIKK polypeptides are novel molecular targets for the development of agents that, in principle, might sensitize cancer cells to conventional chemotherapeutic agents or ionizing radiation. Indeed, wortmannin is an effective radiosensitizing agent in tumor cells. As described herein, wortmannin-induced radiosensitization is manifested at drug concentrations similar to those required for inhibition of DNA-PK and ATM, but not ATR, in A549 lung adenocarcinoma cells. In addition, the clinically proven immunosuppressive and antiproliferative activities of rapamycin validate mTOR as a target for drug discovery efforts. Identification of PHAS-I as an *in vitro* substrate for the phosphatidylinositol kinase-related kinase (PIKK) polypeptides offers a suitable starting point for the implementation of screens for novel inhibitors of this group of protein kinases.

1.0   Methods for Identifying Compounds Inhibiting PIKK Polypeptides

The invention features a method for identifying a compound that inhibits the phosphorylation activity of a PIKK polypeptide. The method includes incubating isolated PIKK polypeptide and a substrate of the PIKK polypeptide with the compound to determine if phosphorylation of the substrate is inhibited. The PIKK polypeptide can be, for example, mTOR, DNA-PK, ATM or ATR. As used herein, "polypeptide" refers to a chain of amino acids of any length. For example, the PIKK polypeptide can be full-length or can be a single domain of the full-length protein, such as a catalytic or kinase domain. In addition, the PIKK polypeptide can be wild-type or mutant. Mutant PIKK polypeptides are

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catalytically active but have decreased binding affinity
for regulatory proteins. For example, deletion of amino
acid residues 2432 to 2449 of mTOR yields a protein with
a 50-100 fold increase in specific activity. Increased
5 specific activity may be desirable for drug screening
protocols.

     mTOR regulates at least two downstream signaling
events in mitogen-stimulated cells. As described herein,
one pathway culminates in the phosphorylation of the eIF-
10 4E binding protein PHAS-1, at serine and threonine
residues involved in the regulation of eIF-4E binding
affinity. The second pathway leads to the
phosphorylation and activation of p70S6 kinase (Brown,
E.J. et al., Nature 369:756 (1994); Sabatini, D.M. et
15 al., Cell 78:35 (1994); Chen J. et al., Proc. Natl. Acad.
Sci. USA 92:4947 (1995)). Both p70S6 kinase and PHAS-I
have been implicated in the coupling of growth factor
receptor occupancy to increases in protein synthesis.
The proposal that mTOR functions as an upstream regulator
20 of the translational machinery receives strong support
from genetic studies in budding yeast. Hall, M.N,
Biochem. Soc. Trans. 24:234 (1996); Barbet, N.C. et al.,
Mol. Biol. Cell 7:25 (1996); DiComo, J.C. et al., Genes
Dev. 10:1904 (1996). These studies have shown that the
25 rapamycin-sensitive, G_1 progression functions of the yeast
TOR proteins are linked to the stimulation of cap-
dependent translation.

     The rate of progression of mammalian cells through
G_1 phase is thought to be governed in part by the ratio of
30 the translational stimulator, eIF-4E, to
hypophosphorylated PHAS-I. The functional consequences
of an imbalance between these positive and negative
regulators of translation are illustrated by the finding
that constitutive overexpression of eIF-4E induces
35 malignant transformation of NIH 3T3 fibroblasts.

Lazaris-Karatzas, A. et al., Nature 345:544 (1990);
Lazaris-Karatzas, A. et al., Genes Dev. 6:1631 (1992);
Rousseau, D. et al., Proc. Natl. Acad. Sci. USA 93:1065
(1996). The transformed phenotype of the eIF-4E-
5 overexpressing cells is partially reversed by genetic
manipulations leading to a counterbalancing increase in
PHAS-I expression in these cells. Rousseau, D. et al.,
Oncogene 13:2415 (1996). Thus, hypophosphorylated PHAS-I
serves as a negative growth regulator in normal cells,
10 and may function as a tumor suppressor in vivo.

Appropriate PIKK polypeptide substrates include
PHAS-1 polypeptide and a peptide substrate from the
amino-terminus of p53. All mammalian members of the PIKK
family can phosphorylate PHAS-I to varying extents.
15 PHAS-1 can be expressed and isolated as described below
or can be obtained from Stratagene (La Jolla, CA).
Although the actual phosphorylation site(s) in PHAS-I are
known only for mTOR, this peptide contains the minimal SQ
kinase recognition motif which appears to be shared by
20 ATM and DNA-PK. While phosphopeptide mapping is
necessary to confirm that these two proteins share this
SQ site as a target for phosphorylation, this overlap in
kinase specificity has been described previously for p53
where both ATM[6] and DNA-PK phosphorylate an SQ site at
25 Ser-15 of p53. The minimal recognition motif for ATR
remains undefined; however, it does not appear to share
the same motif as DNA-PK and ATM since ATR cannot
phosphorylate the N-terminal fragment of p53 containing
Ser-15. The peptide substrate containing this N-terminal
30 fragment of p53 is 15 amino acids in length and is
available from Promega (Madison, WI) or can be chemically
synthesized using standard techniques.

1.1   Isolation of PIKK polypeptides
        In general, PIKK polypeptides can be isolated
35 using techniques known in the art. For example, the

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coding sequence of a PIKK polypeptide can be cloned into
a vector and expressed in a host cell. Vectors contain
suitable regulatory elements to control the expression of
the PIKK polypeptide and are typically a plasmid, cosmid,
5 or a viral vector. Various viral vectors that can be
utilized include adenovirus, herpes virus, vaccinia, or,
preferably, an RNA virus such as a retrovirus.
Preferably, the retroviral vector is a derivative of a
murine or avian retrovirus. Examples of retroviral
10 vectors in which a single foreign gene can be inserted
include, but are not limited to: Moloney murine leukemia
virus, Harvey murine sarcoma virus, murine mammary tumor
virus, and Rous Sarcoma Virus. A number of additional
retroviral vectors can incorporate multiple genes. All
15 of these vectors can transfer or incorporate a gene for a
selectable marker so that transduced cells can be
identified and generated.

Suitable regulatory elements include promoter
nucleic acid sequences, enhancer nucleic acid sequences,
20 inducible elements, transcription termination sequences
or other control sequences. In general, plasmid vectors
contain promoters and control sequences that are derived
from species compatible with the host cell. Promoters
suitable for use with prokaryotic hosts illustratively
25 include the β-lactamase and lactose promoter systems
(Chang, et al., Nature, 275:615, (1978); and Goeddel, et
al., Nature, 281:544, (1979), alkaline phosphatase, the
tryptophan (trp) promoter system (Goeddel, Nucleic Acids
Res., 8:4057 (1980) and hybrid promoters such as the taq
30 promoter (de Boer, et al., Proc. Natl. Acad. Sci. USA,
80:21-25 (1983). However, other functional bacterial
promoters are suitable. Their nucleotide sequences are
generally known in the art, thereby enabling a skilled
worker to ligate them to a polynucleotide encoding the
35 peptide of interest (Siebenlist, et al., Cell, 20:269

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(1980) using linkers or adapters to supply any required restriction sites.

Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from

5 various sources, for example, the genomes of viruses such as polyoma, Simian Virus 40, adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and later promoters of the SV40

10 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers, et al, Nature, 273:113 (1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment

15 (Greenaway, et al., Gene, 18:355-360 (1982). Promoters from the host cell or related species also are useful herein.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase

20 (Hitzeman, et al., J. Biol. Chem., 255:2073 (1980) or other glycolytic enzymes (Hess, et al. J. Adv. Enzyme Reg. 7:149 (1968); and Holland, Biochemistry, 17:4900 (1978) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase,

25 phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other yeast promoters, which are inducible promoters having the additional advantage of transcription

30 controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degraded enzymes associated with nitrogen metabolism, metallothionine, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and

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galactose utilization. Yeast enhancers also are
advantageously used with yeast promoters.

Enhancer elements include the SV40 enhancer on the
late side of the replication origin (bp 100-270), the
5 cytomegalovirus early promoter enhancer, the polyoma
enhancer on the late side of the replication origin, and
adenovirus enhancers. Expression vectors that contain a
gene which operatively encodes a polypeptide and are
intended to be introduced into eukaryotic host cells
10 (yeast, fungi, insect, plant, animal, human or nucleated
cells from other multicellular organisms) will also
contain sequences necessary for the termination of
transcription which may affect mRNA expression. Examples
of suitable selectable markers for mammalian cells which
15 are known in the art include dihydrofolate reductase
(DHFR), thymidine kinase or neomycin. When such
selectable markers are successfully transferred into a
mammalian host cell, the transformed mammalian host cell
can survive if placed under selective pressure (i.e., by
20 being conferred with drug resistance or genes altering
the nutrient requirements of the host cell).

Construction of suitable vectors containing
desired coding, non-coding and control sequences employ
standard ligation techniques. Isolated plasmids or DNA
25 fragments are cleaved, tailored, and relegated in the
form desired to construct the plasmids required. Host
cells may be transformed with the expression vectors of
this invention and cultured in conventional nutrient
media modified as is appropriate for inducing promoters,
30 selecting transformants or amplifying genes. The culture
conditions, such as temperature, pH and the like, are
those previously used with the host cell selected for
expression, and will be apparent to the ordinarily
skilled artisan.

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For purposes of monitoring expression, recombinant
gene expression vectors may be modified to include genes
that operatively encode known reporter polypeptides. For
example, the pRSV lac-Z DNA vector described in Norton,
5   et al., _Mol. Cell. Biol._, 5:281 (1985), may produce β-
galactosidase with protein expression. Luciferase and
chloramphenicol acetyl transferase ("CAT"; see, e.g.,
Gorman, et al., supra, re construction of a pRSV-CAT
plasmid) may also be used. Convenient plasmid
10 propagation may be obtained in _E. coli_ (see, e.g.,
Molecular Cloning: A Laboratory Manual)

Expressed polypeptides can be isolated from host
cells by conventional chromatographic techniques. For
example, gel-filtration, ion-exchange or immunoaffinity
15 chromatography can be used to isolate the proteins.
Reverse-phase high performance liquid chromatography
(HPLC), ion-exchange HPLC, size-exclusion HPLC, or
hydrophobic-interaction chromatography also can be used.
See, for example, "Short Protocols in Molecular Biology",
20 Ed. Ausubel, F.M et al., Greene Publishing Associates and
John Wiley & Sons, 1992, Chapter 10.

Alternatively, PIKK polypeptides can be isolated
by immunoprecipitation. In general, cultured cells are
prepared for lysis by washing in phosphate-buffered
25 saline (PBS) prior to harvesting, then incubating with a
lysis buffer in the cold and homogenizing, for example,
by briefly sonicating. Lysis buffers can include non-
ionic detergents such as Nonidet P-40 (NP-40), Igepal CA-
630 (Sigma), chelating agents such as EDTA and EGTA, or
30 high or low salt concentrations. Proteolytic activity
within a lysate can be minimized by including protease
inhibitors such as aprotinin, pepstatin, leupeptin,
phenylmethylsulfonyl fluoride (PMSF) and microcystin. A
typical lysis buffer can include, for example, 20 mM
35 HEPES buffer, pH 7.4, 1.5 mM $MgCl_2$, 0.15 M NaCl, 1 mM

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EGTA, 1 mM dithiothreitol (DTT), and protease inhibitors.
After lysis, the lysate is cleared by centrifugation in
the cold, i.e. 4°C, at 10,000-12,000 x g for 2-5 minutes,
then diluted to an appropriate protein concentration.

5 Antisera can be added to the lysate and incubated on ice
for about 1 to about 4 hours. Immune complexes can be
precipitated with protein A sepharose beads, then washed
in lysis buffer, kinase buffer and a high salt buffer
(0.6 M NaCl in 100 mM Tris, pH 7,4) prior to assaying.

10 1.2   *Kinase Assays*

Appropriate assay conditions minimally include a
buffer and a phosphate donor such as ATP or GTP. For
example, a phosphorylation assay can include
approximately 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 50 mM

15 NaCl, 10 mM MnCl2, 1 mM dithiotheitol (DTT), 10 mM $[^{32}P]\gamma$
ATP.

Approximately 0.1 μg of PIKK polypeptide and about
1 μg of PHAS-1 can be incubated in the presence of a
compound. If a cell extract is the source of PIKK

20 protein, about 100 μg to about 2000 μg of total protein
is used. Typically, from about 1 nM to about 1 mM of a
compound can be included in the assay. After an initial
screening, the optimal concentration of compound can be
refined by using an entire range of concentrations.

25   Kinase assays are typically incubated from about 5
to about 50 minutes in length and are maintained at about
30°C. For example, the incubation can be from about 15
to about 25 minutes. The length of incubation can be
adapted to the incubation temperature. For example, if

30 the incubation temperature is about 25°C, incubation time
can be increased.

Phosphorylation of PHAS-1 polypeptide can be
monitored in various ways, for example by
electrophoresing the protein mixture through an SDS

35 polyacrylamide gel, which is then dried and exposed to x-

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ray film. The phosphorylation of PHAS-1 polypeptide is
compared with a corresponding assay in the absence of
compound. Alternatively, inhibition of phosphorylation
can be assessed by measuring the amount of radioactivity
5 incorporated into PHAS-1 polypeptide. In this method,
the assay is terminated by addition of an equal volume of
30% acetic acid and then spotted onto P-81
phosphocellulose paper (Whatman LabSales, Hillboro, OR)
or other suitable material. The paper is then rinsed for
10 five minutes with 1% phosphoric acid and 10 mM sodium
pyrophosphate. After four cycles of rinsing,
radioactivity is measured by scintillation counting. The
phosphocellulose paper can also be washed with 30%
trichloroacetic acid (TCA) for 30 minutes at
15 approximately 65°C, then subsequently washed two to four
times with 15% TCA for 15 minutes. After drying, the
filters are washed in ethanol, dried and counted in a
liquid scintillation counter. Casnellie, J.E., Meth.
Enzymol., 200:155 (1991).
20   All the mammalian PIKK family members are
inhibited by wortmannin in the low nM to the low μM
range. While the mechanism of PIKK inhibition by
wortmannin has yet to be defined, it has been extensively
studied in PI3K. Wortmannin covalently binds to Lys-802
25 in the ATP binding domain of PI3K which blocks the
binding of ATP in the catalytic cleft. This lysine
residue is critical for PI3K kinase activity with a
Lys802Arg mutation resulting in a catalytically inactive
enzyme. Based on the conservation of this lysine residue
30 throughout the mammalian PIKK family and the irreversible
binding of wortmannin to the PIKK members demonstrated in
this study, wortmannin may inhibit all PIKK members in a
manner similar to PI3K by binding to this crucial lysine
residue and thereby acting as a non-competitive inhibitor
35 of ATP binding.

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Wortmannin inhibition of DNA-PK has been demonstrated. In contrast to the half-maximal inhibition of DNA-PK at 16 nM as described herein, two other studies have reported IC_{50}s of 250 to 300 nM. These differences

5 in the reported sensitivity of DNA-PK to wortmannin can be explained by the diverse means used for isolating and assaying the DNA-PK catalytic activity. In the study reported below, DNA-PK was immunoprecipitated in a physiologic buffer with mild detergent conditions, which

10 is presumed to preserve the integrity of the DNA-PK heterotrimer. This presumption is supported by the sensitivity of the DNA-PK kinase activity to high salt treatment which has been reported to result in the dissociation of the Ku subunits. Hartley et al. used

15 purified DNA-PK$_{cs}$ in their experiments which is more resistant to inhibition by wortmannin than the intact DNA-PK heterotrimer. Hartley, K.O. et al., Cell, 82:849-856 (1995). In a second study, DNA-PK was not isolated, but assayed directly in cell lysates from SW480 cells

20 treated with wortmannin prior to an in vitro kinase assay. The DNA-PK 'specific' peptide substrate used in the kinase assay was derived from the amino-terminus of p53 and includes Ser-15. As described above, ATM can also phosphorylate Ser-15 of p53. Thus, it is possible

25 that the activity measured in these cells lysates was a combination of DNA-PK and ATM kinase activities.

Inhibition of DNA-PK and ATM kinase activity following incubation of intact A549 cells with low micromolar concentrations of wortmannin correlates with

30 the observed dose-response for radiosensitization in the same cell line. Consistent with inhibition of these kinases, wortmannin caused a marked increase in the initial slope and elimination of the shoulder in the radiation survival curve for A549, suggestive of a defect

35 in post-irradiation DNA repair. Such a defect was

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reported earlier by Boulton et al. who demonstrated that
20 μM wortmannin produced near-maximal inhibition of DNA
double strand break (dsb) repair in Chinese hamster ovary
cells following exposure to ionizing radiation. Boulton,
5 S. et al., Carcinogenesis, 17:2285 (1991). This defect
in DNA dsb repair led to the speculation that inhibition
of DNA-PK was the primary mechanism for wortmannin-
mediated radiosensitization.

However, there are now several lines of evidence
10 to suggest that the inhibition of ATM kinase activity
contributes to radiosensitization by wortmannin. The
demonstration that murine SCID (severe-combined
immunodeficiency) cells, which lack functional DNA-PK,
are radiosensitized by wortmannin first suggested that
15 additional protein target(s) may be involved in
wortmannin-mediated radiosensitization. As shown herein,
inhibition of ATM kinase activity is not only seen at
radiosensitizing concentrations of wortmannin, but is
also associated with multiple cell cycle checkpoint
20 defects. Although the understanding of cell cycle
checkpoints is superficial, it is thought that the
dysfunction of these checkpoints is responsible for the
radiation hypersensitivity observed in AT patients.
Similar to AT cell lines, cells treated with
25 radiosensitizing concentrations of wortmannin prior to
irradiation undergo a prolonged G2 arrest. Further, the
radioresistant DNA synthesis seen following wortmannin
treatment is indicative of an abrogation of a S-phase
checkpoint and is one of the hallmarks of cells derived
30 from AT patients. More importantly, RDS is not seen in
SCID cells which further supports the argument that DNA-
PK is not the only target for wortmannin-mediated
radiosensitization. Thus, inhibition of both ATM and
DNA-PK contribute to the mechanism of radiosensitization
35 by wortmannin.

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Wortmannin represents a lead compound in a novel
class of radiosensitizers which inhibit signal
transduction pathways involved in DNA damage checkpoints
and DNA repair. Traditional radiosensitizers achieve
5 synergistic tumor cell killing by either enhancing the
level of initial DNA damage caused by radiation or
impeding the repair of radiation-induced DNA lesions by
inhibiting enzymes involved in DNA metabolism, synthesis
and repair. In contrast, the inhibition of checkpoint
10 PIKKs would not only impair the proximal signaling
pathways controlling DNA repair, but also would eliminate
crucial cell cycle checkpoints that allow time for DNA
repair to occur.

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2.0   Antibodies

The invention also features an antibody or fragment thereof having specific binding affinity for a conjugate including wortmannin or an analog thereof and a

5 polypeptide. The antibody can be polyclonal or monoclonal. The conjugate can include, for example, wortmannin and a PIKK polypeptide, for example, mTOR, DNA-PK, ATM and ATR.

In general, wortmannin is conjugated to a

10 polypeptide such as ovalbumin and injected into a host mammal. Various host animals can be immunized by injection of a conjugate including wortmannin and a polypeptide. Host animals include rabbits, chickens, mice, guinea pigs and rats. Various adjuvants that can

15 be used to increase the immunological response depend on the host species and include Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole

20 limpet hemocyanin and dinitrophenol. Polyclonal antibodies are heterogenous populations of antibody molecules that are contained in the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous

25 populations of antibodies to a particular antigen, can be prepared using wortmannin conjugated to a polypeptide such as ovalbumin and standard hybridoma technology.

In particular, monoclonal antibodies can be obtained by any technique that provides for the

30 production of antibody molecules by continuous cell lines in culture such as described by Kohler, G. et al., <u>Nature</u>, 256:495 (1975), the human B-cell hybridoma technique (Kosbor et al., <u>Immunology Today</u>, 4:72 (1983); Cole et al., <u>Proc. Natl. Acad. Sci USA</u>, 80:2026 (1983),

35 and the EBV-hybridoma technique (Cole et al., "Monoclonal

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Antibodies and Cancer Therapy", Alan R. Liss, Inc., pp.
77-96 (1983). Such antibodies can be of any
immunoglobulin class including IgG, IgM, IgE, IgA, IgD
and any subclass thereof. The hybridoma producing the
5 monoclonal antibodies of the invention can be cultivated
in vitro and *in vivo*.

Antibody fragments that have specific binding
affinity for a conjugate including wortmannin or an
analog thereof and a polypeptide can be generated by
10 known techniques. For example, such fragments include
but are not limited to F(ab')$_2$ fragments that can be
produced by pepsin digestion of the antibody molecule,
and Fab fragments that can be generated by reducing the
disulfide bridges of F(ab')$_2$ fragments. Alternatively,
15 Fab expression libraries can be constructed. See, for
example, Huse et al., Science, 246:1275 (1989).

Once produced, the antibodies or fragments thereof
are tested for recognition of wortmannin bound to a
polypeptide by Western blotting or immunoprecipitation as
20 described herein. Antibodies of the invention are
particularly useful for deconvoluting the methods for
identifying inhibitors of PIKK proteins. In particular,
the antibodies are useful for preventing the re-isolation
of wortmannin from the screening assays. Alternatively,
25 the antibodies can be used to identify potential target
proteins for wortmannin or analogs thereof in drug-
treated cells.

3.0   *Radioresistant DNA Synthesis*

The invention also features a method for
30 identifying a compound that induces radioresistant DNA
synthesis within cells. The method includes irradiating
cells, wherein the cells include an effective amount of
the compound, and then measuring radioresistant DNA
synthesis of the cell. The presence or absence of

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radioresistant DNA synthesis is then correlated with the
activity of the compound.

Radioresistant DNA synthesis is indicative of an
abrogation of an S-phase checkpoint and is measured in
5 the following manner. Cultured cells in exponential
growth are harvested and plated in 96-well plates. After
approximately 18 hours, cells are irradiated at room
temperature with a sufficient dose rate. The irradiated
cells are treated as indicated with a compound for
10 approximately 20 minutes at 37°C prior to pulsing with ^{3}H-
methyl-thymidine for about 40 minutes. Cells are
harvested, transferred onto glass filters and lysed in
distilled water. Filter-bound radioactivity is then
determined by scintillation counting.
15   Compounds identified as inducing radioresistant
DNA synthesis can be further assessed to determine if
they inhibit the phosphorylation activity of a PIKK
polypeptide using the above-described methodology.

The invention will be further described in the
20 following examples, which do not limit the scope of the
invention described in the claims.

Examples

Example 1 - General Methods: Cell Culture,
Antisera and Wortmannin Treatment: Human embryonic
25 kidney 293 cells (HEK 293) were maintained in DMEM
supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2
atmosphere at 37°C. K562 erythroleukemia cells were
maintained in RPMI 1640 with 10% FBS. The A549 lung
adenocarcinoma cell line was maintained in RPMI 1640
30 (GibcoBRL) containing 10% FBS. A fibroblast cell line,
GM02052, derived from an AT patient, was obtained from
the Coriell Institute for Medical Research. GM02052 was
maintained in minimal essential medium (GibcoBRL) with 15
mM HEPES, 20% FBS, and 2x non-essential and 2x essential

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amino acids (GibcoBRL). Wortmannin (Sigma) was stored at
-80°C as a 20 mM stock solution in dimethylsulfoxide
(DMSO) and diluted immediately prior to use in either
RPMI 1640 for treatment of intact cells or in aqueous
5 buffer. Diluted wortmannin was added directly to the
media and incubated at 37°C for one hour prior to lysis
for all kinase assays and immunoblotting experiments
involving drug treatment of intact cells.

Wortmannin-specific monoclonal antibodies were
10 generated in the Mayo Department of Immunology Monoclonal
Antibody Facility by immunizing Balb/c mice with
wortmannin conjugated to ovalbumin. The resulting
hybridoma supernatants were screened for specificity by
an enzyme-linked immunosorbent assay with wortmannin
15 conjugated to bovine serum albumin. On the basis of
these initial tests, one antibody-producing hybridoma,
designed Wm7.1, was selected for further evaluation. Rat
brain extracts were treated with drug vehicle or with 1
μM wortmannin and subjected to SDS-PAGE and
20 immunoblotting with Wm7.1. The results showed that Wm7.1
specifically reacted with a subset of proteins from
wortmannin-treated rat brain extracts, but not with
control brain extracts. Wm7.1 used in subsequent
experiments was purified from ascites fluid by affinity
25 chromatography over Protein G-Sepharose (Pharmacia).

Polyclonal antibodies were generated against the
same immunogen in New Zealand White rabbits.
Immunizations, boost and antibody bleeds were performed
commercially by Cocalico, Inc. Polyclonal antisera were
30 screened for wortmannin-specific immunoreactivity as
described above. Rabbit polyclonal antiserum specific
for ATR and antibodies specific for ATM (Ab-3) and DNA-PK
(Ab-1) were obtained from Oncogene Research-Calbiochem.

Cloning The cDNAs encoding wild type mTOR (mTOR-
35 wt), a rapamycin-resistant mTOR mutant (mTOR-rr), and the

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rapamycin-resistant, kinase-dead mTOR double mutant
(mTOR-kd) were inserted as restriction fragments into the
polycloning region of pcDNA3 (Invitrogen). The Ser^{2035}→Ile
substitution that generated the mTOR-rr mutant and the
5 Ser^{2035}→Ile, Asp^{2338}→Ala substitution that generated the
mTOR-kd double mutant were created using the Transformer
kit (Clontech, Palo Alto, CA). The cDNAs were tagged at
their 5'-termini with nucleotide sequences encoding the
six amino acid sequence recognized by monoclonal antibody
10 AU1 (Babco, Richmond, CA). The PHAS-I expression vector,
pCMV4-PHAS-I is described by Lawrence, J., Adv. Enzyme
Regul., 37:239-267 (1997).

     Transfections Transfections were performed using
TransIT polyamino transfection reagent (Pan Vera
15 Corporation, Madison, WI) according to manufacturer's
instructions. Approximately 2 μg pCMV4-PHAS-I and 4 μg
of pcDNA3 or pcDNA3 vectors encoding wild-type or mutant
mTOR proteins were used to transfect HEK 293 cells seeded
into 60 mm dishes at 5 x 10^5 cells per dish.
20 Alternatively, K562 erythroleukemia cells (10^7 cells per
sample) were transfected using a BTX model T820 square-
wave electroporator. The cells were mixed with 25 μg
pcDNA3-mTOR or pcDNA3-mTOR-kd plasmid DNA plus 20 μg
pcDNA3 only as filler DNA. Mock transfections were
25 performed with 45 μg pcDNA3 only. The cells were
electroporated with a single pulse at field settings of
350 V and 10 msec duration. For p38 MAP kinase assays,
K562 cells were transfected with FLAG-p38-encoding
plasmid. Control transfections (Co) were performed with
30 pcDNA3 only.

     Expression of the transfected mTOR mutants and the
phosphorylated forms of PHAS-I was determined by
immunoblotting equivalent amounts of cellular protein
with AU1 mAb and anti-PHAS-I antibodies, respectively.

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Immunoblotting of mTOR and PHAS-1   Transfected
cells were cultured for 12 to 16 hours in appropriate
medium supplemented with 10% FBS.   HEK 293 cells were
cultured in DMEM supplemented with 10% FBS for 12 hours,
5 then transferred into low-serum medium containing 0.1%
FBS and then cultured for an additional 12 hours, at
which time selected samples received either 5 nM
rapamycin, the indicated concentrations of wortmannin, or
drug vehicle only.   The cells were treated for 30 to 60
10 minutes in culture, and then recombinant human insulin
(Gibco BRL, Grand Island, NY) was added to final
concentration of 100 nM.

After an additional 30 minutes, cell monolayers
were washed with PBS, and harvested by scraping into
15 lysis buffer (50 mM β-glycerophosphate, 1.5 mM ethylene
glycol-bis(β-aminoethyl ether)N,N,N',N',-tetraacetic acid
(EGTA), pH 7.4, supplemented with 0.5 mM Na_3VO_4, 20 nM
microcystin-LR, 0.2mM PMSF, 10 μg/ml leupeptin, 5 μg/ml
aprotinin, 5 μg/ml pepstatin, and 1% Nonidet P-40 (NP-
20 40)).   Alternatively, cells were osmotically shocked for
10 minutes with 0.4 M sorbitol, then disrupted by
sonication in lysis buffer (50 mM Tris HCl, 50 mM β-
glycerophosphate, 100 mM NaCl, pH 7.4, 10% glycerol, 1 mM
Na_3VO_4, 1 mM DTT, 0.2% Tween-20, and the standard cocktail
25 of phosphatase and protease inhibitors).   Post-nuclear
detergent extracts were equalized for protein content and
were mixed with reducing SDS-PAGE sample buffer.

Duplicate samples were electrophoresed through
12.5% and 7.5% polyacrylamide gels for PHAS-I and AU1 mAb
30 immunoblots, respectively.   PHAS-I immunoblots were
performed with affinity-purified rabbit polyclonal
antibodies generated against a peptide derived from the
carboxy-terminus of PHAS-I.   Lin, T.A., and Lawrence,
J.C., J. Biol. Chem., 271:30199 (1996).   AU1-tagged mTOR
35 polypeptides were blotted with AU1 mAb followed by rabbit

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anti-mouse IgG antibodies (Pierce). Recombinant p38 was
immunoprecipitated with anti-FLAG mAb M1 (Eastman Kodak).
The amount of p38 in the anti-FLAG immunoprecipitates was
assessed by immunoblotting with a p38-specific antibody
5 (New England Biolabs). Immunoreactive proteins were
detected with horseradish peroxidase coupled to protein A
followed by chemiluminescence detection using the ECL
reagent (Amersham).

Immunoprecipitation and Blotting of ATM, ATR and
10 DNA-PK Late log-phase A549 cells in 100 mm tissue
culture dishes were washed twice with PBS and then lysed
on ice with scraping in lysis buffer (20 mM Hepes, pH
7.4, 0.15 M NaCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT, 10
μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20
15 nM microcystin) containing 0.5% NP-40. Lysates were
cleared by centrifugation, pooled if appropriate, and
diluted to 2-3 mg/ml. For immunoprecipitations, 2 μl
ATR, 8 μg ATM (Ab-3, catalog No. PC#116, Oncogene
Research, Calbiochem) and 3 μg DNA-PK antisera were added
20 to 1 ml of lysate and incubated on ice for 1.5 to 2
hours. Fifteen μl packed Protein A-Sepharose beads were
added and tubes were rotated for an additional 30 to 60
minutes at 4°C. Immunoprecipitates were washed twice in
lysis buffer and twice in kinase base buffer (10 mM
25 Hepes, 50 mM NaCl, 10 mM MgCl$_2$, pH 7.4) prior to
incubation with graded doses of wortmannin. Samples were
subjected to SDS-PAGE and transferred to Immobilon-PVDF
membranes (Millipore) prior to immunoblotting.
Wortmannin-bound proteins were detected by probing the
30 membrane with Wm7.1 at 0.8 μg/ml in Tris-buffered saline
containing 0.02% Tween-20 (TBST) and 5% milk overnight at
4°C. After washing in TBST, membranes were incubated
with a secondary polyclonal rabbit anti-mouse IgG
antibody (Pierce). PIKK members were blotted with the
35 appropriate antisera diluted in 5% milk/TBST for 1-2

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hours at room temperature. Blots were developed with
horseradish peroxidase coupled to protein A and the
enhanced Chemiluminescence reagent (Amersham).

Immune Complex Kinase Assays The ATM kinase assay
5 was a modification of a previously described method.
A549 cells were lysed for 20 minutes as described above,
with the exception that 0.2% Tween-20 was substituted for
NP-40 as the detergent and lysates were diluted to 0.5
mg/ml. In experiments involving the AT fibroblasts,
10 cells were harvested by trypsinization and lysates were
sonicated to maximize the yield of nuclear proteins.
Equivalent amounts of protein (0.5 mg) were incubated on
ice for 2 hours with ATM-specific antibodies and
precipitated with potential sepharose beads. Following
15 immunoprecipitation, immune complexes were washed twice
in lysis buffer with DTT, phosphatase and protease
inhibitors, once in a high salt buffer (0.6 M NaCl/0.1 M
Tris, pH 7.4) and once in kinase base buffer. When
indicated, immunoprecipitates were incubated with graded
20 concentrations of wortmannin for 30 minutes in the dark
at room temperature. The kinase reaction mix was then
added to give a final concentration of 10 mM Hepes, 50 mM
NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 mM [γ-^{32}P]ATP
(specific activity: 50 Ci/mmol; ICN) and 25 ng/μl PHAS-I
25 (Stratagene) in a total volume of 40 μl. Kinase
reactions were incubated at 30°C for 20 minutes. The
phosphorylation of appropriate substrates by each kinase
followed linear kinetics under the reaction conditions
described. Reactions were terminated with the addition
30 of an equal volume of 30% acetic acid and duplicate
aliquots were spotted onto P-81 phosphocellulose paper
(Whatman). The papers were rinsed for five minutes with
1% phosphoric acid/10 mM sodium pyrophosphate. After
four cycles of rinsing, the radioactivity retained on the
35 paper was measured by scintillation counting.

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The DNA-PK kinase assay was similar to the ATM
kinase assay with the exception that the lysates were
sonicated prior to clearing, and the protein
concentration was adjusted to 0.75 mg/ml prior to
5   immunoprecipitation with anti-DNA-PK antibodies. The
immune complexes were washed twice with lysis buffer and
twice with kinase base buffer prior to the kinase
reaction. The kinase reaction conditions were identical
to those described above with the exceptions that the
10   reaction time was 15 minutes at 30°C and the substrate
was a 15 amino acid DNA-PK peptide substrate derived from
p53 (Promega, catalog #V5811). The DNA-PK substrate was
used at a concentration of 250 ng/μl per reaction.
Samples spotted onto P-81 paper were rinsed with four 5
15   minute cycles in 15% acetic acid/10 mM sodium
pyrophosphate prior to liquid scintillation counting. In
experiments evaluating the sensitivity of ATM and DNA-PK
kinase activity to high salt, immunoprecipitates were
washed twice with lysis buffer and then with either
20   kinase base buffer or a high salt buffer (0.6 M NaCl/0.1
M Tris, pH 7.4). All immunoprecipitates were then washed
with kinase base buffer before addition of the kinase
reaction mix. In these experiments, PHAS-I was used as
the peptide substrate in both the ATM and the DNA-PK
25   kinase reactions.

Cellular extracts for immunoprecipitation of ATR
were prepared with the lysis buffer containing 1% Triton
X-100. Lysates were diluted to 2 mg/ml protein for
immunoprecipitation. The ATR kinase assay was identical
30   to the ATM kinase assay, except the kinase reaction was
run for 15 minutes at 30°C and was stopped by the
addition of an equal volume of 4x SDS-PAGE loading
buffer. Samples were separated by SDS-PAGE, and the
proteins were transferred onto Immobilon-PVDF membranes.

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The incorporation of ^{32}P into PHAS-I was quantitated with
an AMBIS 4000 imaging system.

    The protein kinase activity of native mTOR was
assayed by immunoprecipitation of this protein from rat
5 brain extracts. Sabers, C.J. et al., J. Biol. Chem.,
270:815 (1995). The extracts were supplemented with 1 mM
DTT, 0.2 μM microcystin LR, and 10 μg/ml each of
leupeptin, pepstatin, and aprotinin. The extracts (1 mg
protein per sample) were mixed with affinity-purified
10 rabbit polyclonal antibodies directed against a peptide
sequence corresponding to residues 2432-2449 of mTOR.
The immune complexes were precipitated with 15 μl protein
A-Sepharose beads and the immunoprecipitates were washed
two times in immunoprecipitation buffer (50 mM Tris HCl,
15 50 mM β-glycerophosphate, 100 mM NaCl, pH 7.4, 10%
glycerol, 20 nM microcystin LR, 10 μg/ml leupeptin, 5
μg/ml aprotinin, 5 μg/ml pepstatin A, and 600 μM PMSF).
The precipitates were washed one time in high-salt buffer
(100 mM Tris HCl, pH 7.4, 500 mM LiCl), followed by two
20 washes in kinase buffer.

    The immunoprecipitates were pretreated with 25 μl
of kinase buffer containing 10 μg glutathione S-
transferase (GST)-FKBP12 fusion protein and, where
indicated, 10 μM rapamycin or 100 μM FK506. Parallel
25 samples were pretreated in a similar fashion with various
concentrations of wortmannin. After 40 minutes at room
temperature, the beads were washed two times in kinase
buffer.

    p38 kinase activity toward PHAS-I was assayed as
30 described in Karnitz, L.M. et al., Mol. Cell. Biol.,
15:3049 (1995). The amount of p38 in the anti-FLAG
immunoprecipitates was assessed by immunoblotting with a
p38-specific antibody (New England Biolabs). The
immunoprecipitates were treated with GST-FKBP12 plus
35 rapamycin (F•R) or FK506 (F•FK), or with 1 μM wortmannin

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(Wm).   Immune complex kinase assays were performed with
PHAS-I as the substrate. ^{32}P-labelled PHAS-I was detected
by autoradiography.   Expression of recombinant mTOR
proteins was detected by immunoblotting with AU1
5   monoclonal antibody.   The immunoprecipitated FLAG-p38 was
detected by immunoblotting with a p38-specific antibody.
          Clonogenic Assay   The effect of wortmannin on the
radiosensitivity of A549 cells was assessed with a
clonogenic assay.   A549 cells in log-phase were
10   harvested, resuspended in fresh growth medium and plated
in triplicate in 60 mm dishes at cell concentrations
estimated to result in 20-100 colonies per dish following
treatment.   Four hours after plating, cells were
irradiated at room temperature with ^{137}Cs source at a dose
15   rate of 6.4 Gy/min.   Wortmannin was added to the
indicated samples immediately after irradiation.   The
final concentration of the drug solvent did not exceed
0.1% (vol/vol), and this solvent concentration had no
effect on either the clonogenicity or radiosensitivity of
20   the A549 cells.   Cells were cultured for two weeks prior
to fixation and staining with Coomassie Blue.   Only
colonies with greater than 50 cells were scored.   Data
shown represent the mean of four independent experiments
with error bars representing the standard error of the
25   mean (SEM).
          Cell Cycle analysis   A549 cells were synchronized
at the G_1/S border by culturing for 18 hours in 5 μg/ml
aphidicolin (Sigma).   Dishes were washed once with PBS
and fresh medium was added.   At 3.5 hours after release
30   from the aphidicolin block, the S-phase-enriched cells
were treated with wortmannin.   Thirty minutes later,
cells were irradiated with 0 or 5 Gy as described above.
After an additional 30 minutes, drug-containing medium
was replaced with fresh culture medium.   Twenty-four
35   hours later, cells were harvested by trypsinization,

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fixed in PBS containing 70% ethanol, and the samples were
stored at -20°C. The fixed cells were resuspended in PBS
containing 20 μg/ml propidium iodide and 100 μg/ml boiled
RNaseA, and were incubated from 30 minutes at 37°C for 30

5   minutes prior to flow cytometric analysis on a Becton-
Dickinson FACScan. Twenty-thousand ungated events were
collected. Cell cycle distribution was determined with
the ModFit software package (Verity) after excluding
doublets and clumps by gating on the DNA pulse height

10   versus pulse area displays.

        Radioresistant DNA Synthesis A549 cells in
exponential growth were harvested and plated in 96-well
plates (10,000 cells per well in 0.1 ml). Each treatment
condition was tested in 6 replicate wells. After 18

15   hours, the cells irradiated at room temperature at a dose
rate of 6.4 Gy/min. The irradiated cells were treated as
indicated with wortmannin for 20 minutes at 37°C prior to
the pulsing with 2 μCi per well ^{3}H-methyl-thymidine
(specific activity: 5 Ci/mmol; Amersham) for 40 minutes.

20   Cells were harvested by trypsinization, transferred onto
glass filters and lysed in distilled water. Filter-bound
radioactivity was determined by scintillation counting.
Data shown represent the mean of three independent
experiments with error bars representing the standard

25   error of the mean (SEM).

        Statistics All statistical analyses were
performed using the software program Sigma Plot 4.0
(SPSS). The concentrations resulting in half-maximal
inhibition (IC_{50}) for the various kinases were calculated

30   by fitting the data sets with the Hill 4-parameter
equation, using a least-squares regression, and then
solving the equations for a relative activity of 0.5.
The radiation dose-response curves were fit with the
linear-quadratic equation: $\ln(S) = -\alpha D - \beta D^2$, where S is

35   the surviving fraction, D is the dose of radiation, and α

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and β are constants. Fowler, J.F., <u>Br. J. Cancer</u>,
49:285-300 (1984). The sensitizer enhancement ratio
(SER) for each survival curve was calculated as the ratio
of radiation doses, that resulted in 10% survival of the
5 cells in the absence or presence of wortmannin. A
paired, two-tailed student T-test was used to determine
the statistical difference between the calculated SER at
each dose of wortmannin as compared to no drug treatment.

<u>Example 2 - Effect of a rapamycin-resistant mTOR</u>
10 <u>mutant on insulin-stimulated PHAS-I phosphorylation</u>: To
test whether or not mTOR is an upstream component of the
PHAS-I phosphorylation pathway, HEK 293 cells were
cotransfected with expression vectors encoding rat PHAS-I
and either wild-type (mTOR-wt) or a rapamycin-resistant
15 mTOR (mTOR-rr) mutant that contains a single amino acid
substitution ($S^{2035} \rightarrow I^{2035}$) within the FKBP12•rapamycin-
binding (FRB) domain. Chen, J. et al., <u>Proc. Natl. Acad.
Sci.</u>, 92:4947 (1995). This substitution generated a
catalytically active version of mTOR that bears a lower
20 binding affinity for the FKBP12•rapamycin complex. To
facilitate detection of the wild-type and mutant mTOR
cDNA products, both polypeptides were appended at their
5'-termini with a tag sequence recognized by the AU1
monoclonal antibody (mAb). After transfection, the cells
25 were rested in low-serum medium, then treated for 30
minutes with rapamycin prior to stimulation for 30
minutes with insulin. Changes in the phosphorylation
state of PHAS-I were monitored indirectly by
immunoblotting. Previous studies have shown that
30 phosphorylation of PHAS-I decreases the electrophoretic
mobility of this protein during SDS-PAGE. Lin, T.A. et
al., <u>Science</u>, 266:653 (1994). Rapamycin treatment
inhibited the insulin-stimulated phosphorylation of PHAS-
I in mock-transfected or mTOR-wt-transfected 293 cells,
35 as indicated by the decrease in immunoreactivity of the

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most highly phosphorylated form of PHAS-I, and by the
appearance of hypophosphorylated PHAS-I. In contrast,
rapamycin caused no detectable decrease in PHAS-I
phosphorylation in mTOR-rr-expressing 293 cells. The
5 ability of the mTOR-rr transfectants to maintain PHAS-I
in a hyperphosphorylated state following exposure to the
drug suggests that a rapamycin-sensitive activity of mTOR
is required for insulin-stimulated PHAS-I phosphorylation
in intact cells.

10   The addition of FKBP12•rapamycin to immune complex
kinase assays containing mTOR inhibits the
autophosphorylating activity of this kinase *in vitro*.
Brown, E. J. et al., <u>Nature</u>, 377:442 (1995). Therefore,
a functional mTOR catalytic domain may be needed for the
15 phosphorylation of mTOR in intact cells. As a first
approach to test this hypothesis, the impact of
wortmannin on PHAS-I phosphorylation in HEK 293 cells
cotransfected with either mTOR-wt or the mTOR-rr mutant
was assessed. Although wortmannin has been widely used
20 as an inhibitor of PI 3-kinase, this drug also
irreversibly inhibits the autophosphorylating activity of
mTOR. Brunn, G.J. et al., <u>EMBO J.</u>, 15:5256 (1996). The
concentration of wortmannin required to inhibit mTOR
autokinase activity by 50% (IC_{50}) was approximately 200
25 nM, and this activity was maximally inhibited by 1 μM
wortmannin. Because wortmannin targets the ATP-binding
site of mTOR, rather than the FRB domain, the drug can
nondiscriminately block the kinase activities of both
wild-type mTOR and the rr-mTOR mutant. This prediction
30 was borne out by the finding that 1 μM wortmannin
strongly inhibited the phosphorylation of PHAS-I in
insulin-stimulated 293 cells transfected with either
mTOR-wt or the mTOR-rr mutant. In contrast, pretreatment
of the transfected cells with 0.1 μM wortmannin, a drug
35 concentration sufficient to fully inhibit endogenous PI

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3-kinase activity (Karnitz, L.M. et al., <u>Mol. Cell Biol.</u>, 15:3049 (1995)), failed to suppress the phosphorylation of PHAS-I. The sensitivity of insulin-stimulated PHAS-I phosphorylation to wortmannin is consistent with the idea

5 that a functional mTOR kinase domain is required for this phosphorylation event *in vivo*.

<u>Example 3 - Role of mTOR kinase activity in PHAS-I phosphorylation within intact cells</u> Additional genetic evidence for the involvement of the mTOR kinase domain in

10 PHAS-I phosphorylation was supplied by transfection experiments with a kinase-dead version of the mTOR-rr mutant. The mTOR-rr/kd double mutant contains the rapamycin resistance-conferring $S^{2035} \to I$ substitution described above, together with a $D^{2338} \to A$ substitution that

15 inactivates the mTOR kinase domain (Brunn, G.J. et al., <u>EMBO J.</u>, 15:5256 (1996)). The 293 cells were cotransfected with PHAS-I and a mTOR-rr/kd expression vector. Control cell populations were cotransfected with pcDNA3 only, or with the pcDNA3 vector encoding the

20 catalytically-active, mTOR-rr mutant. Rapamycin largely eliminates the contribution of endogenous mTOR to the phosphorylation of the transfected PHAS-I protein, and allows a direct comparison of the capacities of the catalytically-active and -inactive mTOR mutants to

25 support this response in intact cells. Whereas PHAS-I was predominantly hyperphosphorylated in mTOR-rr-expressing 293 cells, extensive dephosphorylation of PHAS-I was evident in cells transfected with the catalytically-inactive mTOR-rr/kd double mutant. The

30 decrease in PHAS-I phosphorylation induced by rapamycin treatment of mTOR-rr/kd-expressing cells was similar to that observed in the mock-transfected control cells. These results support the notion that an intact kinase domain is required for the participation of mTOR in the

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pathway leading to PHAS-I phosphorylation in intact
cells.

      **Example 4 - Phosphorylation of PHAS-I by
immunopurified native mTOR**   Previous studies have
5 demonstrated that mTOR displays serine-specific
autokinase activity in immune complex kinase assays.
Brunn, G.J. et al., EMBO J., 15:5256 (1996); Brown, E.J.
et al., Nature, 377:442 (1995).   To investigate the
possibility that PHAS-I itself might serve as a substrate
10 for mTOR, immune complex kinase reactions were performed
with mTOR immunoprecipitates from rat brain extracts and
recombinant PHAS-I as the substrate.   The addition of [γ-
^{32}P]ATP to the mTOR-containing kinase reactions resulted
in the incorporation of radiolabeled phosphate into PHAS-
15 I, as well as the appearance of more slowly migrating
forms of the protein after separation by SDS-PAGE.   In
contrast, non-immune IgG immunoprecipitates contained no
detectable phosphorylating activity toward PHAS-I.   The
PHAS-I kinase activity present in mTOR immunoprecipitates
20 was strongly inhibited by the addition of
FKBP12•rapamycin to the *in vitro* kinase reaction, whereas
FKBP12•FK506 had no effect on the phosphorylation of
PHAS-I.   Furthermore, pretreatment of the
immunoprecipitated mTOR with wortmannin suppressed the
25 PHAS-I kinase activity at drug concentrations (0.1 - 1
μM) identical to those required for inhibition of mTOR
autophosphorylation *in vitro*.   Table 1 presents the
amount of [^{32}P]-incorporation into PHAS-I, normalized to
that measured in the non-drug treated control.

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Table 1

Wortmannin (nM)	$[^{32}P]$-incorp.
0	100
30	145
100	98
300	56
1000	8

The sensitivity of this phosphorylation reaction to
rapamycin and wortmannin strongly supports to the
conclusion that mTOR itself is responsible for the
phosphorylation of PHAS-I in the immune complex kinase
assays.

In subsequent studies, the abilities of
recombinant wild-type or kinase-inactive versions of mTOR
to phosphorylate PHAS-I *in vitro* were compared. The
recombinant proteins were prepared by transfecting K562
erythroleukemia cells with either the mTOR-wt expression
plasmid or a plasmid encoding a kinase-dead mutant mTOR
(mTOR-kd) bearing the inactivating $D^{2338} \rightarrow A$ substitution in
the catalytic domain. After transfection, the AU1-tagged
mTOR-wt and mTOR-kd proteins were immunoprecipitated from
K562 cell extracts with AU1 mAb. As was observed with
the native, rat brain-derived mTOR, recombinant mTOR-wt
phosphorylated PHAS-I in immune complex kinase assays.
Phosphoamino acid analysis indicated that the
phosphorylation occurred on both serine and threonine
residues in PHAS-I. In contrast, the level of PHAS-I
phosphorylation catalyzed by AU1 immunoprecipitates from
mTOR-kd-transfected cells was indistinguishable from the
background activity obtained with immunoprecipitates from
mock-transfected cells. Moreover, the PHAS-I kinase
activity found in mTOR-wt immunoprecipitates was strongly

inhibited by FKBP12•rapamycin or wortmannin, but not by
FKBP12•FK506.

In a parallel control experiment, K562 cells were
transfected with an expression vector encoding a FLAG
5   epitope-tagged version of the p38 MAP kinase, which, like
ERK1 and ERK 2 (Lin, T.A. et al., _Science_, 266:653
(1994); Haystead, T.A.J. et al., _J. Biol. Chem._,
269:23185 (1994)), phosphorylates PHAS-I _in vitro_. The
FLAG-tagged p38 was immunoprecipitated from cell
10 extracts, and the immunoprecipitates were treated with
FKBP12•rapamycin, FKBP12•FK506, or wortmannin prior to
the immune complex kinase assay. Although recombinant
p38 readily catalyzed the phosphorylation of PHAS-I _in
vitro_, p38 kinase activity was not inhibited by
15 FKBP12•rapamycin or 1 μM wortmannin. These results argue
that both FKBP12•rapamycin and wortmannin inhibit the
PHAS-I kinase activity present in mTOR immunoprecipitates
by selectively and directly inhibiting the catalytic
activity of mTOR itself.

20   **Example 5 - Phosphorylation of PHAS-I by
recombinant, immunopurified mTOR-wt, mTOR-kd protein and
FLAG-tagged p38** The effect of mTOR-mediated
phosphorylation on the eIF-4E binding activity of PHAS-I
was examined by Far Western analysis. Recombinant PHAS-I
25 was incubated with control antibody or anti-mTOR antibody
immunoprecipitates from rat brain extracts. Immunoblot
analysis of the kinase reaction products demonstrated
that exposure to mTOR generated 3 electrophoretically
distinct forms of phosphorylated PHAS-I. Pretreatment of
30 the mTOR immunoprecipitates with FKBP12•rapamycin or 1 μM
wortmannin blocked both the phosphorylation of PHAS-I and
the appearance of PHAS-I forms with retarded
electrophoretic mobilities. In contrast, PHAS-I
phosphorylation was not inhibited by either rapamycin or
35 FK506 alone, or by the FKBP12•FK506 complex. The same

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sample lanes were probed with ^{32}P-labeled eIF-4E in a Far
Western blot. Phosphorylation of PHAS-I by mTOR strongly
inhibited the eIF-4E-binding activity of PHAS-I in this
assay. In accordance with the PHAS-I phosphorylation
5 results described above, the inhibitory effect of mTOR-
mediated phosphorylation on PHAS-I binding to eIF-4E was
blocked by FKBP12•rapamycin or 1 μM wortmannin (Figure
1). Taken together, these results demonstrate that the
phosphorylation of PHAS-I on serine and threonine
10 residues by mTOR downregulates the eIF-4E-binding
activity of PHAS-I.

     Example 6 - ATM Kinase Assay: Before assessing
the sensitivity of the various PIKKs to wortmannin, a
series of preliminary experiments were performed to
15 document the specificity of the ATM immune complex kinase
assay. A549 cell extracts were immunoprecipitated with
preimmune serum or with ATM-specific antibodies as
described in Example 1. Samples were then pretreated for
30 minutes at room temperature with 1 μm wortmannin.
20 Immune complex kinase reactions were performed as
described in Example 1 and the reaction products were
separated by SDS-PAGE. The amount of ^{32}P$_i$ incorporation
into PHAS-1 substrate was measured with a Molecular
Dynamics Phosphorimager System and ImageQuant Software.
25 The results of this experiment are depicted in Table 2.
ATM immunoprecipitates from A549 cells phosphorylated
PHAS-I to an approximately 25-fold higher level than that
catalyzed by control antibody immunoprecipitates.

Table 2

30

Preimmune Serum	ATM	ATM + Wortmannin
1.0	4.6	0.6

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Extracts prepared for an ATM-negative fibroblast
line (GM02052) were immunoprecipitated with anti-ATM
antibodies. The lack of a detectable kinase activity in
ATM immunoprecipitates from the AT fibroblast line
5 GM02052 demonstrated that the kinase activity observed
was from ATM or a kinase physically associated with ATM
(Figure 2A). To evaluate the possibility of co-
precipitation of other PIKKs in the ATM immune complex,
α-ATM immunoprecipitates were immunoblotted for DNA-PK or
10 ATR. No DNA-PK or ATR immunoreactivity was detected in
the ATM immunoprecipitates. Moreover, the DNA-PK and ATR
immunoprecipitates were similarly free of contamination
by other PIKK family members. The stability of the ATM
kinase activity against PHAS-I, when exposed to a high
15 salt wash, further documented the lack of a contaminating
kinase activity in the ATM immune complex. In contrast,
DNA-PK phosphorylation of PHAS-I was significantly
reduced after a high salt wash, consistent with the
dissociation of the DNA-PK heterotrimer of DNA-PK$_{cs}$, Ku70
20 and Ku80 (Figure 2B). Thus, based on the absence of ATM
kinase activity in an ATM null cell line, the lack of co-
association between ATM and either DNA-PK or ATR, and the
integrity of the ATM kinase after a high salt wash, it
was concluded that the kinase activity seen in ATM
25 immunoprecipitates is due to the intrinsic kinase
activity of ATM.

Example 7 - Wortmannin Binds PIKK Protein: To
determine the relative potency of wortmannin as an
inhibitor of the catalytic activities of DNA-PK, ATM and
30 ATR, appropriate PIKK immunoprecipitates were treated
with various concentrations of wortmannin prior to the
immune complex kinase assays. As seen in Figure 3A-C,
the sensitivities of the PIKKs to wortmannin varied over
a wide range, with an IC$_{50}$ of 150 nM for ATM, 16 nM for
35 DNA-PK and 1.8 μM for ATR, respectively. Based on the

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covalent interaction of wortmannin with a critical lysine
residue in the ATP-binding pocket of PI3K, wortmannin may
also inhibit PIKKs by a similar mechanism. To test this
hypothesis, a monoclonal antibody (Wm7.1) that
5 specifically recognizes wortmannin bound to protein was
developed. Using this antibody, wortmannin binding to
DNA-PK, ATM, and ATR was detected by immunoblotting PIKK
immunoprecipitates which had been incubated with various
concentrations of wortmannin. Moreover, the
10 concentrations of wortmannin at which binding was
detected correspond with those necessary for kinase
inhibition. These results are highly suggestive that the
kinase activities of this PIKKs are indeed inhibited by a
covalent interaction with wortmannin.
15   __Example 8 - Inhibition of PIKKs by Wortmannin:__
Because of the irreversible inhibition of these kinases
by wortmannin, it was possible to evaluate the kinase
activity of the PIKKs following incubation of intact
cells with wortmannin. This allows a direct correlation
20 between the inhibition of these proteins by wortmannin in
intact A549 cells with the dose-response for wortmannin-
mediated radiosensitization in these same cells.
Following incubation of intact cells with wortmannin, the
kinase activities of both ATM and DNA-PK were almost
25 completely inhibited at 30 μM (Figures 4A-B) and were
half-maximally inhibited at 5.8 and 3.6 μM, respectively.
As expected, minimal binding of wortmannin was detectable
by immunoblotting with Wm7.1 following treatment of cells
with drug concentrations less than 10 μM. As suggested
30 from the immune complex data, ATR kinase activity was
much more resistant to wortmannin in intact cells (Figure
4C), with an IC_{50} of >100μM and the detection of only
minimal wortmannin binding on Wm7.1 immunoblots following
treatment with wortmannin at concentrations less than 100
35 μM. As a further test of the specificity of protein

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kinase inhibition by wortmannin, A549 cells were treated
with wortmannin, and then cyclin-Cdc2 complexes were
immunoprecipitated from cellular extracts. The histone
H1 kinase activity of Cdc2 was not affected by exposure
5 of A549 cells to 30 mM wortmannin.

Example 9 - Radiosensitization of Cell Lines

Wortmannin has been shown to sensitize a number of human
tumor cell lines to radiation. In the A549 cell line
used in this study, radiosensitization by wortmannin
10 follows a steep dose-response relationship (Figure 5)
with no radiosensitization seen at 2 μM and significant
radiosensitization at 20 μM wortmannin. The degree of
radiosensitization can be expressed as the ratio of
radiation doses that results in a 10% survival,
15 (sensitizer enhancement ratio SER_{10}) which was 2.2 at 20
μM (p=0.02), 1.4 at 10 μM (p=0.05) and 1.0 at 2 μM
wortmannin (p=0.43). Treatment with 30 μM wortmannin did
not result in a significant additional increase in
radiosensitivity as compared to 20 μM. Thus,
20 radiosensitization occurs at concentrations of wortmannin
(10-30 μM) which correspond to the inhibition of ATM and
DNA-PK, but not ATR kinase activities.

Wortmannin not only increased the sensitivity of
A549 cells to radiation, but it also significantly
25 changed the shape of the radiation survival curve.
Compared to the DMSO control, 20 μM wortmannin resulted
in an eight-fold increase in the initial slope, α, as
described by the linear quadratic equation. This
increase in α is suggestive of inhibition DNA repair
30 processes that result in the conversion of potentially
repairable DNA lesions into non-repairable lesions. Also
consistent with inhibition of repair, wortmannin
treatment also resulted in a significant flattening of
the shoulder of the radiation survival curve which is
35 reflected by an increase in the α/β ratio from 2.0 to

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19.5 Gy.   The α/β ratio is the dose at which the linear
(αD) and quadratic (βD^2) contribution to cell killing are
equal and describes how quickly a survival curve begins
to bend.

5   If ATM contributed to the radiosensitizing effect
of wortmannin in A549 cells, then the prominent cell
cycle checkpoint defects exhibited by AT-deficient cells
should be recapitulated in wortmannin-exposed A549 cells.
Cells treated with 20 μM wortmannin prior to irradiation

10 showed a marked accumulation of G$_2$-m-phase cells at 24
hours post-irradiation while non-treated cells displayed
a minor increase in the number of G$_2$-m-phase cells,
indicative of a transient G$_2$ arrest, followed by re-entry
into the cell cycle (Figure 6). This prolonged G2-delay

15 is consistent with inhibition of DNA-PK or ATM since
cells defective in either kinase exhibit a similar G2
delay. ATM deficient, but not DNA-PK deficient cells,
have a defective S-phase checkpoint(s) which results in
one of the hallmark characteristics of AT cells:

20 radioresistant DNA synthesis (RDS). As seen in Figure 7,
the dose-response relationship for induction of RDS in
A549 cells treated with wortmannin correlates with both
the inhibition of ATM kinase activity and
radiosensitization.

25   Other Embodiments
          It is to be understood that while the invention
has been described in conjunction with the detailed
description thereof, the foregoing description is
intended to illustrate and not limit the scope of the

30 invention, which is defined by the scope of the appended
claims. Other aspects, advantages, and modifications are
within the scope of the following claims.

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What is claimed is:

1. A method for identifying a compound inhibiting the phosphorylation activity of a phosphoinositide 3-kinase related kinase polypeptide comprising incubating isolated phosphoinositide 3-kinase related kinase polypeptide and a substrate of said polypeptide with said compound to determine if phosphorylation of said substrate is inhibited.

2. The method of claim 1, wherein said polypeptide is mTOR.

3. The method of claim 1, wherein said polypeptide is ataxia-telangiectasia mutated protein.

4. The method of claim 1, wherein said polypeptide is Ataxia and Rad3 related protein.

5. The method of claim 1, wherein said substrate is PHAS-I protein.

6. The method of claim 1, wherein said compound induces radioresistant DNA synthesis in irradiated cells containing said compound.

7. An antibody or fragment thereof having specific binding affinity for a conjugate comprising wortmannin or an analog thereof and a polypeptide.

8. The antibody of claim 7, wherein said polypeptide is mTOR.

9. The antibody of claim 7, wherein said polypeptide is DNA-dependent protein kinase.

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10.   The antibody of claim 7, wherein said polypeptide is ataxia-telangiectasia mutated protein.

11.   The antibody of claim 7, wherein said polypeptide is Ataxia and Rad3 related protein.

5   12.   The antibody of claim 7, wherein said antibody is polyclonal.

13.   The antibody of claim 7, wherein said antibody is monoclonal.

14.   A method for identifying a compound that
10 induces radioresistant DNA synthesis within cells, comprising:
       (a)   irradiating said cells, wherein said cells comprise an effective amount of said compound;
       (b)   measuring radioresistant DNA synthesis of
15 said cell; and
       (c)   correlating the presence or absence of said radioresistant DNA synthesis with activity of said compound.
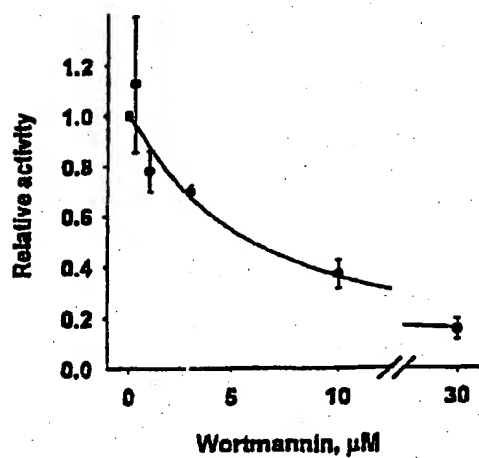
FIGURE 1

FIGURES 2A–B
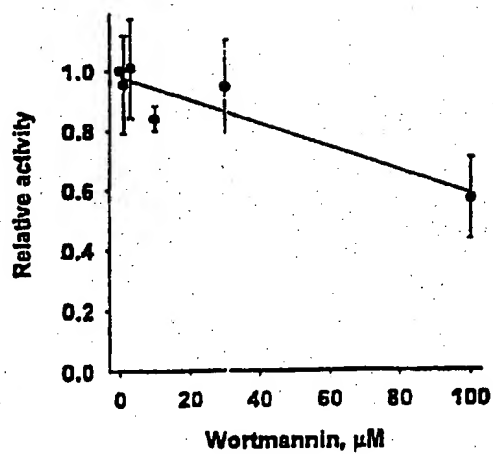
A

B

A

B

C

FIGURE 5

FIGURE 6

Red fluorescence (DNA content)
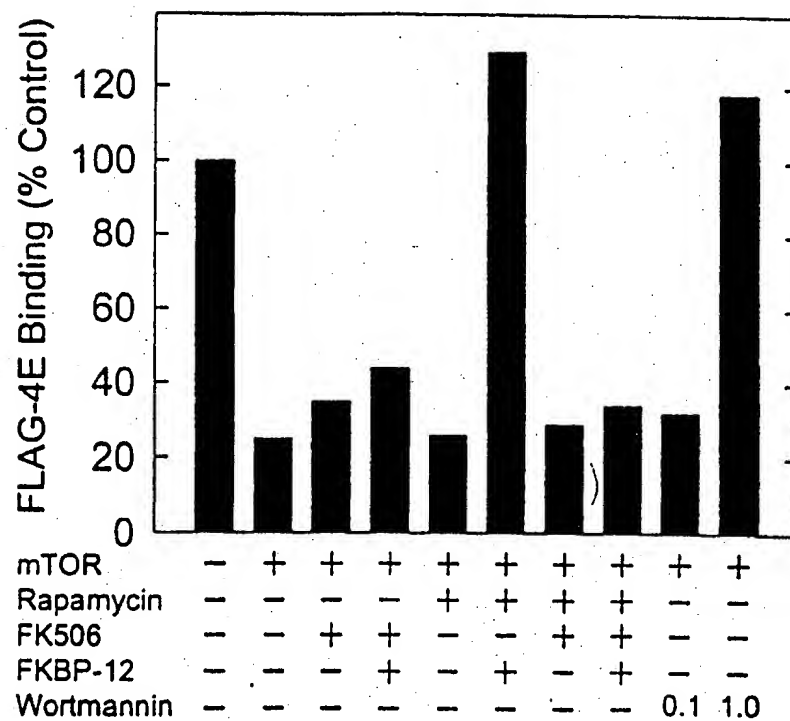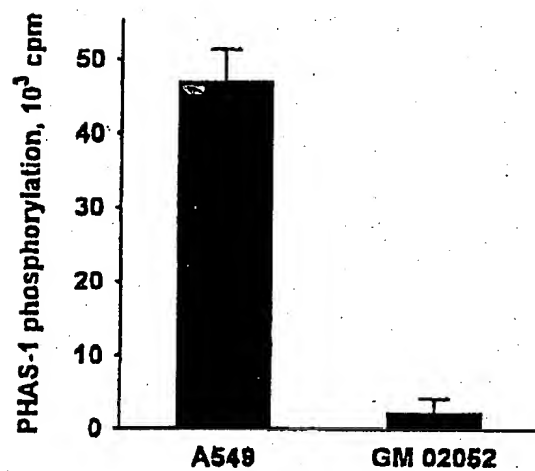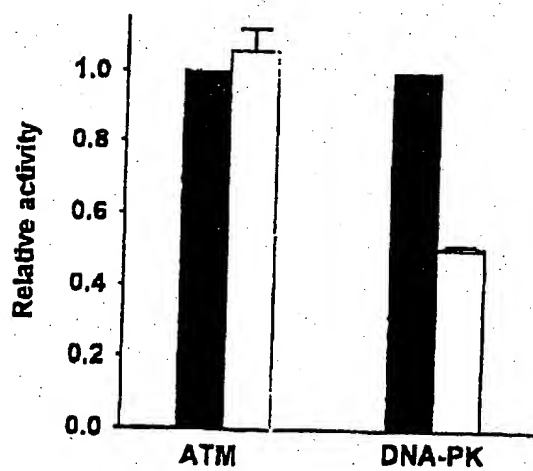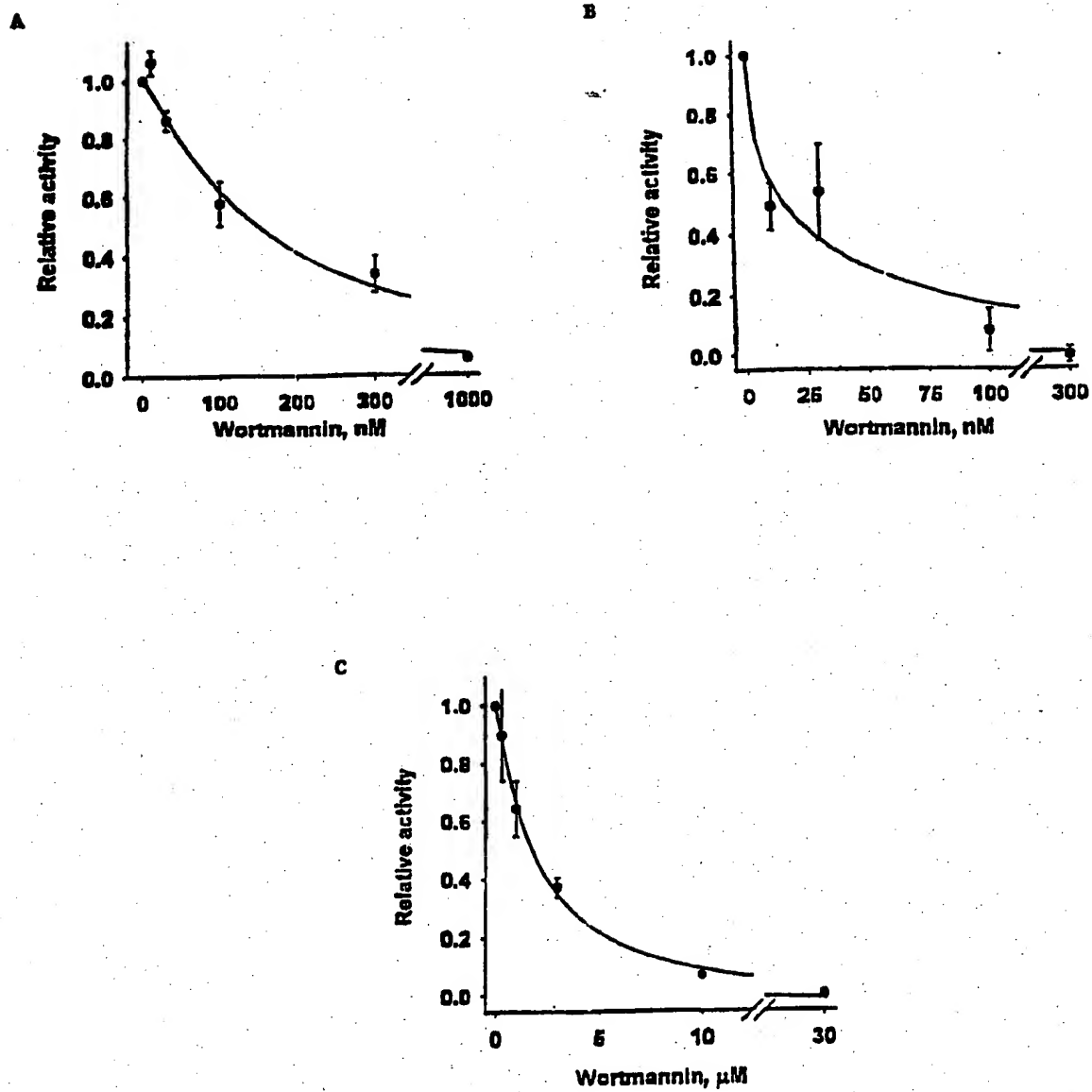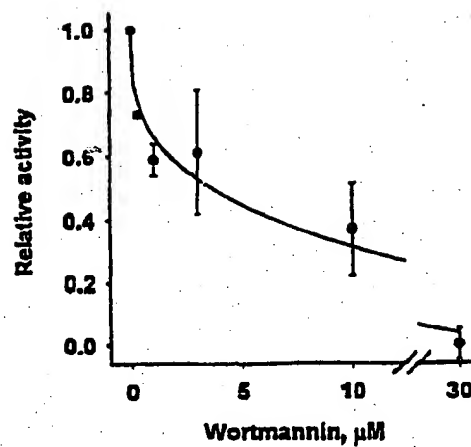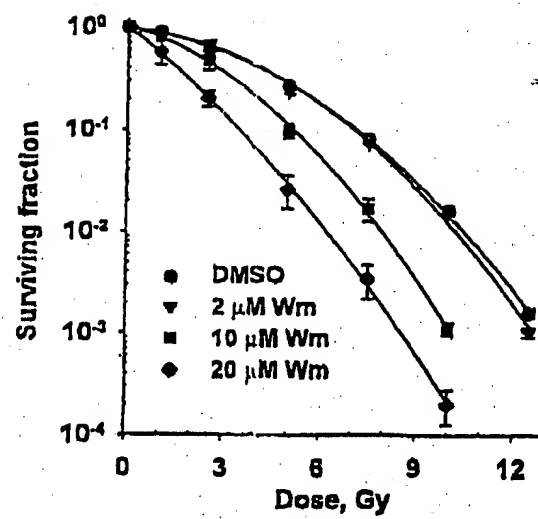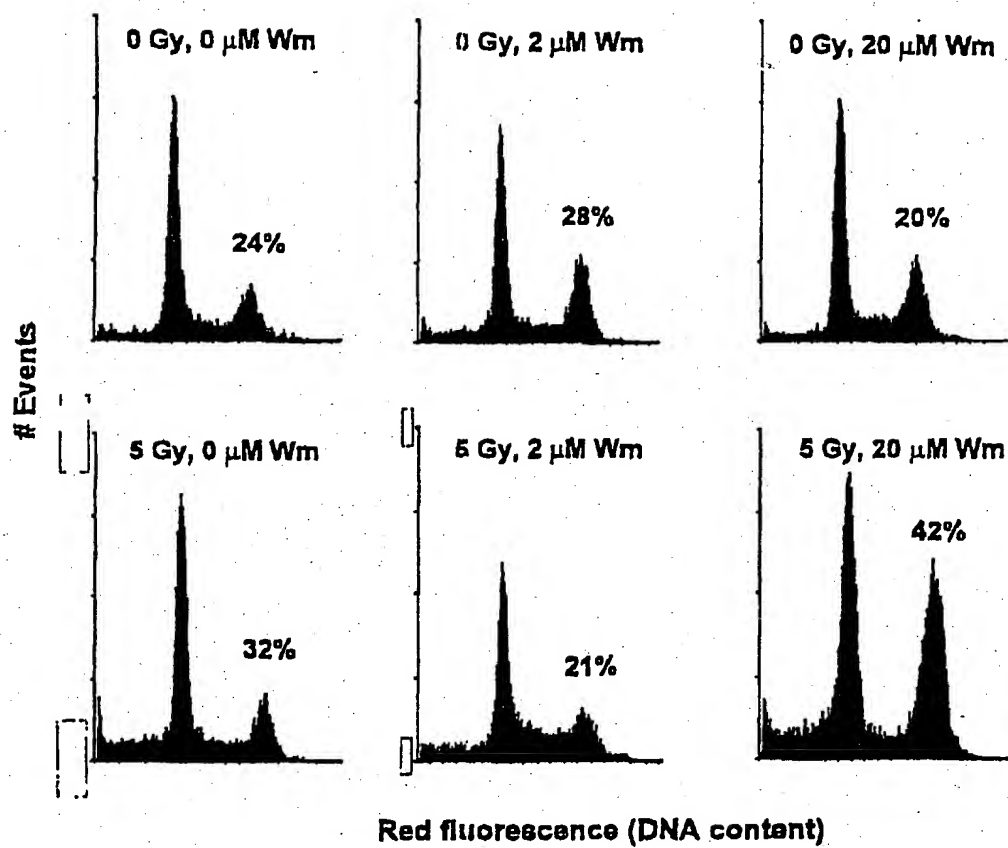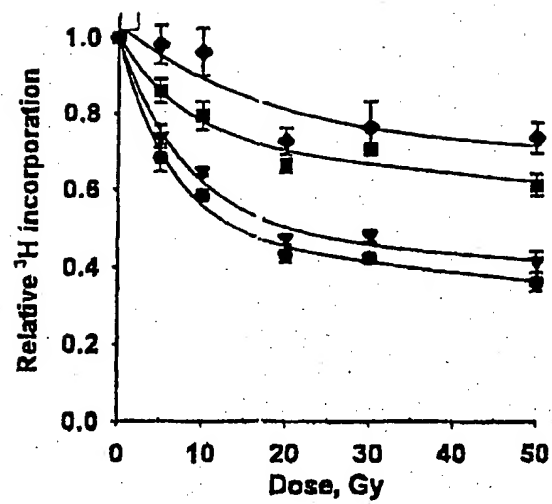
FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11420

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/12; C12Q 1/48, 1/50

US CL   :435/194, 15, 17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/194, 15, 17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,466,716 A (IGARASHI et al) 14 November 1995, see entire document.	1-14
A	US 5,151,360 A (HANDA et al) 29 September 1992, see entire document.	1-14

☐ Further documents are listed in the continuation of Box C.　　☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search	Date of mailing of the international search report
03 AUGUST 1998	0 3 SEP 1998
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